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Familial breast cancer accounts for 15 to 35% of all breast cancers. Mutations in a number of genes are now known to cause susceptibility to breast cancer; the most notorious are the BRCA1 and BRCA2 genes. However, it has become evident that not all (or even the majority) of familial breast cancer families can be attributed to mutations in BRCA1 and BRCA2. In a recent study by the Breast Cancer Linkage Consortium, only one third of families with four or five cases of female breast cancer and no cases of ovarian cancer carry mutations in either BRCA1 and BRCA2. Smaller familial clusters are much more common than families with large numbers of cases, suggesting that a sunstantial proportion of familial clustering is not accounted for by mutations in BRCA1 and BRCA2; therefore, there is a great need to discover other genes that contribute to this disease. We hypothesize that a heterozygous deletion in constitutive DNA or a homozygous deletion in multiple tumors and tumor types from a cancer-prone family will represent a strong candidate cancer predisposing gene. To establish this proof of principle, we have successfully developed a fluorescent-bassed DNA microarray assay to identify deletions, as small as a single exon, in heterogeneous tumor DNA.

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INTRODUCTION:

Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some 6 to 7 years ago, when the breast-cancer susceptibility genes, *BRCA1* and *BRCA2* were identified through positional cloning. Epidemiological studies sparked by the discovery of *BRCA1* and *BRCA2* have made clear several features of inherited mutations in the genes. The mutations are highly penetrant carrying a lifetime risk of 30 to 85% for cancer incidence, with variation related to genetic background. Thus, identifying individuals predisposed to disease is of central importance towards preventive health strategies. One of the emerging themes in cancer risk assessment has been the enormity of the task of screening for genetic susceptibility. Recent studies have suggested that mutations in *BRCA1* and *BRCA2* are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups including ours are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depend on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. To establish a method to effectively detect homozygous deletions in candidate breast cancer susceptibility genes we derived an exon chip and a fluorescent-based DNA microarray assay to identify deletions in tumor DNA.

Body.

Fifteen invasive breast tumors and 15 malignant epithelial tumors of the ovary were evaluated for genomic rearrangements using custom *BRCA1* and *BRCA2* exon DNA microarrays. The tumor DNA samples were compared to control DNA, which was previously shown to be wild-type for *BRCA1* and *BRCA2*. All sample tumor DNAs were paired with control DNA and hybridized to *BRCA1* and *BRCA2* exon DNA array, containing fragments of 24 exons of the *BRCA1* gene and 27 exons of the *BRCA2* gene. Of the 30 tumors evaluated for genomic rearrangements involving *BRCA1* and *BRCA2*, one (UPN 54) revealed a statistically confident 4.45 ± 0.44 ($2^{2.1668}$)-fold difference in signal intensity from the exon 17 fragment, compared to a mean-fold difference of 0.88 ± 0.175 for all other exons in **Figure 1A**. This observation was confirmed in a "dye-flip" experiment, where the sample originally labeled with Cy5 was labeled with Cy3, and vice versa. We performed a statistical analysis of the repeated "dye-flip" experiments to evaluate the reproducibility of measurements and calculated the CVs for intensity ratios and for individual channels to evaluate the reproducibility of the replicate spots on the array. We also performed a statistical confidence analysis to omit the data points with less than a 95% probability of having a different signal due to copy number and not experimental conditions, and we were left with only the one ratio–intensity comparison of exon 17. This confidence analysis is a modified version of the method described by Kerr and Churchill (Kerr et al., 2000) and implemented in the GeneSight 3.0.4 software package.

We also used Southern blot analysis to evaluate the 15 tumor samples for gross genomic rearrangements of the *BRCA1* locus. Of the DNAs evaluated, only UPN 54 showed a larger aberrant migrating DNA fragment when digested with *Eco*RI and hybridized with a cDNA probe spanning exons 15-20 of *BRCA1* (**Figure 2**), due to loss of an *Eco*RI site. As can be seen in **Figure 2,** both the 9,764 bp and 5,730 bp bands in sample UPN54 containing exons 17-19 and exons 15 and 16, respectively, are greatly reduced in intensity. This region of interest was further refined using a panel of restriction endonucleases and the corresponding normal DNA isolated from peripheral-blood leukocytes to determine the nature and extent of the alteration. Tumor-specific additional bands were readily apparent

in DNA digested with *Eco*RI, *Pst*I, and *Sst*I (**Figure 3**). A slightly smaller migrating band was evident in the *Hind*III digested tumor DNA lane as compared to normal. However, the alteration in the *Bam*HI digested sample could not be detected because of the difficulty in resolving this large DNA fragment (>23kbp). In addition, the intensity of the normal bands was reduced in the tumor sample (C) compared to the corresponding normal DNA (N). Based on these digests and a restriction map of the *BRCA1* gene (GenBank accession no. L78833), we determined that the deletion was approximately 3kb in length and included an *Eco*RI site, a *Pst*I site, and two *Sst*I sites in intron 16 of *BRCA1*, all of exon 17, and a portion of intron 17. Long-range PCR further confirmed the size and boundaries of the deletion involving exon 17 (data not shown).

To confirm that the 3-kb deletion detected in the ovarian tumor from UPN 54 resulted in a frameshift and thus expression of a truncated protein product, Western blot analysis was performed (**Figure 4**). The 220-kDa wild-type BRCA1 protein was detected in HeLa cells that contained the wild-type and in two other ovarian tumors (UPNs 0698 and 2061). A smaller protein product was detected in the ovarian tumor from UPN 54, showing that the mutation does indeed result in a truncated product and that the protein is expressed. Interestingly, Western blot analysis detected no full-length BRCA1 protein in the heterogeneous tumor tissue (**Figure 4**). Thus, inactivation of *BRCA1* in this tumor from UPN 54 conforms to Knudson's "2-hit" hypothesis of inactivating tumor suppressor genes.

Overall, we have found genomic rearrangements affecting the *BRCA1* gene in a small percentage (7%; 1/15) of the ovarian adenocarcinomas evaluated with a rapid *BRCA1* and *BRCA2* exon DNA array approach. No mutations were discovered in breast tumors in either *BRCA1* or *BRCA2*. Nevertheless, our work has shown that the method we have developed can simultaneously search for DNA rearrangements in the *BRCA1* and the *BRCA2* genes. Furthermore, it can be expanded and applied to the search for large rearrangements in a variety of genes of interest simultaneously. Even though the sensitivity of the method has not been rigorously tested, we have shown that a deletion in a tumor containing ~30% normal DNA could be detected readily. Overall, the method presented has implications for the rapid and simultaneous evaluation of multiple cancer-associated genes in clinical tumor samples as well as in the germline of high-risk families without apparent *BRCA1* or *BRCA2* mutations for large genomic rearrangements.

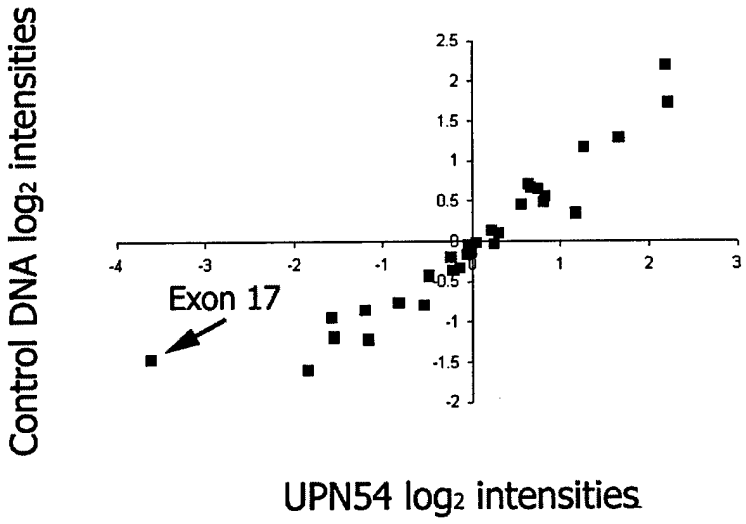

Figure 1. *BRCA1* exon DNA array analysis of UPN 54 tumor DNA showing a deletion of exon 17 of *BRCA1*. Scatter plot of hybridization $\log_2$ signal intensities from *BRCA1* exons from affected sample UPN 54 (X-axis) and unaffected control UPN 58 sample (Y-axis). Arrow indicates the position of $\log_2$ hybridization intensities from exon 17.

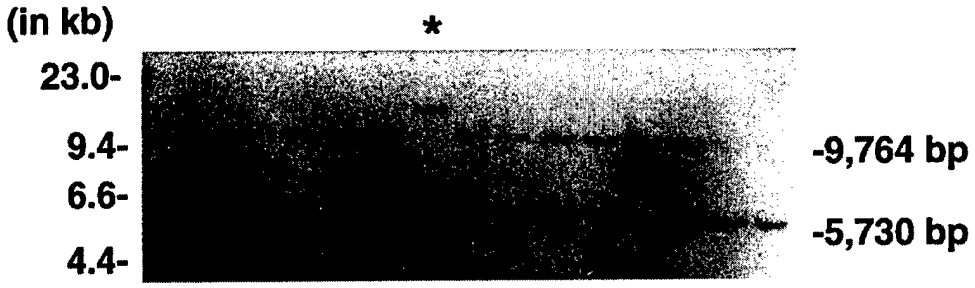

Figure 2. Southern blot analysis of 15 tumor DNAs for genomic rearrangements in *BRCA1*. Genomic tumor DNA was digested with *Eco*RI, separated on a 1% agarose gel, transferred to a nylon membrane, and probed with a ~700 bp cDNA fragment of *BRCA1* spanning exons 15 through 20. Asterisk indicates UPN 54 sample showing an aberrant migrating DNA fragment.

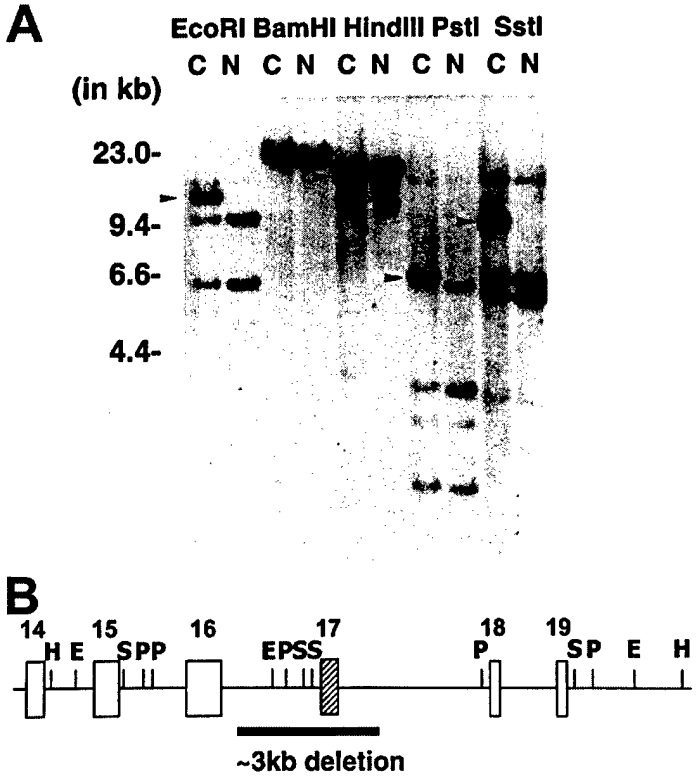

Figure 3. Southern blot analysis of UPN54. (A) DNA from patient UPN 54 was extracted from blood lymphocytes (N=normal) or tumor (C=cancer). Restriction enzymes used to digest the DNA are indicated above the panels. The blot was hybridized with an ~700 bp probe containing exons 15-20 of *BRCA1* cDNA. Molecular weights (in kb) are to the left of the blot; arrow heads indicate variant bands in the tumor sample. (B) Restriction digest map: boxes represent exons; H, *Hind*III; E, *Eco*RI; S, *Sst*I; and P, *Pst*I. The relative position of the genomic deletion is represented below the digestion map.

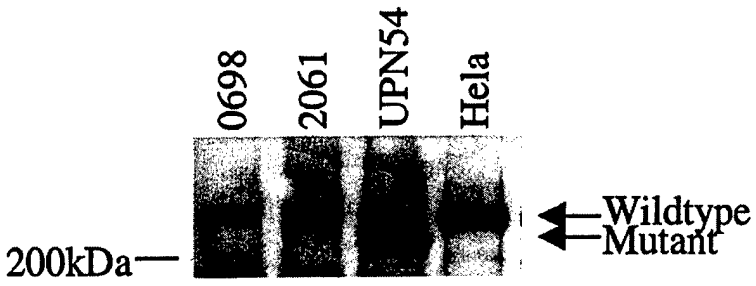

Figure 4. Western blot analysis of UPN 54 for mutant BRCA1 protein. Extracts from the indicated samples were separated by 6% SDS-PAGE and processed by Western blotting. The blot was probed with the anti-BRCA1 antibody. Lanes 0698, 2061 extracts of sporadic ovarian tumors with wild-type BRCA1; lane UPN 54 extract of sporadic ovarian tumor from UPN54 showing a truncated BRCA1 protein due to deletion of exon 17; lane HeLa, extract from the cervical carcinoma cell line with wild-type BRCA1.

C- KEY RESEARCH ACCOMPLISHMENTS:

C.1. "Identification of candidate breast cancer susceptibility genes using a cDNA microarray/CGH approach".

1.a. Successfully fabricated a *BRCA1* and a *BRCA2* exon DNA array.

1.b. Developed a fluorescent-based DNA microarray assay to identify deletions, as small as a single exon, in heterogeneous tumor DNA

1.c. Identified that exon 17 of *BRCA1* was deleted in an ovarian tumor sample.

1.d. Confirmed the presence of the deletion by Southern and Western analysis.

1.e. Published the method in Genes, Chromosomes, and Cancer and presented this method at the 10^{th} Annual SPORE meeting in Washington and a cancer workshop in Seattle.

1.f. Received funding from the Army to continue the studies which were supported by this concept mechanism, i.e., "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene", DAMD17-03-1-0312.

D-REPORTABLE OUTCOMES (1/2002 to present):

***D.1.*.** "Identification of candidate breast cancer susceptibility genes using a cDNA microarray/CGH approach".

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1.a. Abstracts

Querec, T.D., Gruver, B.N., Patriotis, P.C., Stoyanova, R.S., Frolov, A.E., Engstrom, P.F., **Godwin, A.K.,** Brown, T.R., Patriotis, C. Differential gene expression patterns associated with the in vitro malignant transformation of human ovarian epithelial cells and chemopreventitive treatment with fenretinide. Proceedings of American Association of Cancer Research, 609:3277, 2001.

Prowse, A.H., Salicioni, A.M., Dunbrack, R., **Godwin, A.K.** OVCA1, a candidate tumor suppressor, interacts with RBM8: a highly conserved RNA-binding protein. Second International Conference: Proteins that Bind RNA, Austin, TX, pg 116:97, March 4-8, 2001,.

Grobelny, J.V., Lynch, H., **Godwin, A.K.**, Broccoli, D. *BRCA1* Mutations and Telomerase Activity in Breast And Ovarian Tumors. Telomeres and Telomerase, Cold Spring Harbor, March 28-April 1, pp 58, 2001

Prowse, A.H., Salicioni, A.M., Dunbrack, R., **Godwin, A.K.** OVCA1, a candidate tumor suppressor, interacts with RBM8: a highly conserved RNA-binding protein. The Six Annual Postdoctoral Research Conference, Philadelphia, 2001.

Pan, Z-Z., Bruening, B., Giasson, B.I., Lee, V. M. -Y., and **Godwin, A. K**. Gamma-synuclein, a candidate oncogene, interacts with MAPKs and contributes to the metastatic spread of breast and ovarian cancer. The Six Annual Postdoctoral Research Conference, Philadelphia, 2001, oral presentation.

Querec, T.D., Gruver, B.N., Patriotis, P.C., Stoyanova, R.S., Frolov, A.E., Engstrom, P.F., **Godwin, A.K.,** Brown, T.R., Patriotis, C. Differential gene expression patterns associated with the in vitro malignant transformation of human ovarian epithelial cells and chemopreventitive treatment with fenretinide. The Ninth Annual SPORE Workshop, Washington, D.C., 2001, p63, July 2001, oral presentation.

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Pan, Z-Z., Bruening, B., Giasson, B.I., Lee, V. M. -Y., and **Godwin, A. K.** Gamma-synuclein, a candidate oncogene, activates RAC and ERK and contributes to the metastatic spread of breast and ovarian cancer. The American Journal of Human Genetics, 69:521a, 2001.

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osmotic shock: implications for changes in mRNA processing to stress. The American Journal of Human Genetics, 69:456a, 2001.

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Caslini, C., Frolov, A., **Godwin, A.K.**, Broccoli, D. Gene expression profiling of ALT-positive human ovarian surface epithelial cells reconstituted for telomerase activity. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002.

Frolov, A., Arnoletti, J.P., Pan, Z.Z., vonMehren, M., Ochs, M., Eisenberg, B., **Godwin, A.K.** Sprouty 4A; a Novel Diagnostic Marker of Response to Gleevec (STI-571) in Gastrointestinal Stromal Tumors. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002. (Second place, best oral presentation).

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Pan, Z-Z., Bruening, W., Giasson, B.I., Lee, V.M., **Godwin, A.K.** γ-Synuclein may render cancer cells resistant to paclitaxel and nitric oxide induced apoptosis by modulating MAPK pathways. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002.

Pan, Z-Z., Cartledge, D., **Godwin, A.K.** Gamma-synuclein, a candidate oncogene, can promote ovarian tumor cell survival and inhibit stress- and chemotheray drug-induced apoptosis. 4th Biennial Ovarian Cancer Research Symposium, Seattle, WA, Sept 19-20, 2002 (Oral presentation, travel award).

*Frolov, A., Prowse, A. H., Vanderveer, L., Bove, B., Favorova, O., **Godwin, A.K.** A novel DNA array-based method for detection of large rearrangements in the *BRCA1* gene. 4th Biennial Ovarian Cancer Research Symposium, Seattle, WA, Sept 19-20, 2002 (Oral presentation, travel award).

*Ross, E.A., Klein-Szanto, A.J.P., Godwin, K.A. Resource cores to support and promote research within and among SPORE Institutions. In: National Cancer Institute 10th SPORE Investigators' Workshop, July 2002, Chantilly, VA.

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A Frolov, JP Arnoletti, ZZ Pan, J Fletcher, O Favorova, M von Mehren, B Eisenberg, and **A.K. Godwin**. Sprouty 4A; a Novel Diagnostic Marker of Response to Gleevec (STI-571) in Gastrointestinal Stromal Tumors. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002. (Society of Surgical Oncology 56th Annual Cancer Symposium, L.A., CA March 5-9, 2003. Oral presentation).

A Frolov, JP Arnoletti, ZZ Pan, J Fletcher, S. Chahwan, J. Fletcher, O. Favorova, M. von Mehren, B. Eisenberg, and **A.K. Godwin**. Genocentric approach to determine response markers to Gleevec using an *in vitro* model for gastrointestinal stromal tumors (GISMD Anderson Cancer Center 2nd Gastrointestinal Cancer Research Conference, Orlando, November 20-23rd, 2002, 5a (travel award).

D. E. Bassi, R. Lopez De Cicco, P. Alexander, **A.K. Godwin**, and A. Klein-Szanto. Elevated Furin expression in ovarian tumors. Proceedings of American Association of Cancer Research, 44:3369, 2003 .

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Shan-Chun Guo and **A.K. Godwin**. Accumulation of p84N5 domain protein is associated with an aggressive phenotype of human breast tumors. Proceedings of American Association of Cancer Research, 44:2421, 2003.

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Okamoto, I., Tsuiki, H., Kenyon, L.C., **Godwin, A.K.**, Emlet, D.R., Holgado-Madruga, M., Lanham, I.S., Joynes, C.J., Vo, K.T., Guha, A, Matsumoto, M., Ushio, U., Saya, H., and Wong, A.J. Proteolytic cleavage of CD44 adhesion molecule in multiple human tumors. Proceedings of American Association of Cancer Research, 44:4090, 2003.

Corrado Caslini, Alex J. Carlisle, **Andrew K. Godwin** and Dominique Broccoli. BRCA binding to telomeric DNA in ALT-positive cell lines. Telomeres and Telomerase, Cold Spring Harbor, submitted, 2003.

1.b. Publications

Guerardel, C., Deltour, S., Pinte, S., Monte, D., Begue, A., **Godwin, A.K.**, and LePrince, D. Identification in the human candidate tumor suppressor gene HIC-1 of a new major alternative TATA-less promoter positively regulated by p53. J. Biol. Chem., 276:3078-3089, 2001.

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1.c. Invited articles

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E-CONCLUSIONS:

E.1. "Identification of candidate breast cancer susceptibility genes using a cDNA microarray/CGH approach".

In most families with multiple cases of breast and ovarian cancer, the cancer appears to be associated with germline alterations in *BRCA1* or *BRCA2*. However, somatic mutations in *BRCA1* and *BRCA2* in sporadic breast and ovarian tumors are rare, even though loss of heterozygosity in *BRCA1* and *BRCA2* loci in these tumors appear frequently. This may be attributed to mutation detection assays that detect alterations in the coding regions and splice site junctions, but that miss large gene rearrangements. To look specifically for mutations such as large gene rearrangements that span several kilobases (kb) of genomic DNA, we have developed a fluorescence DNA microarray assay. This assay rapidly and simultaneously screens for such rearrangements along the entire gene. In our screen of malignant breast and ovarian tumors, we found one sample with a novel 3-kb deletion encompassing exon 17 of *BRCA1* that leads to a frameshift mutation. This deletion was not detected in the corresponding constitutive DNA. Our results indicate that the method described in this report has the potential to screen clinical tumor samples for genomic rearrangements simultaneously in a large number of cancer-associated genes.

F. LIST OF PERSONNEL PAID FROM GRANT

Godwin, Andrew K.

G. REFERENCES

None

H. APPENDICES

None